Coinfection and Emergence of Rifamycin Resistance during a Recurrent Clostridium difficile Infection

Stevenson, Emma; Kuehne, Sarah; Major, Giles; Spiller, Robin; Minton, Nigel P

DOI:
10.1128/JCM.01025-16
10.1128/JCM.01025-16

Document Version
Peer reviewed version

Citation for published version (Harvard):
Stevenson, E, Kuehne, S, Major, G, Spiller, R & Minton, NP 2016, 'Coinfection and Emergence of Rifamycin Resistance during a Recurrent Clostridium difficile Infection', *Journal of Clinical Microbiology*, vol. 54, no. 11, pp. 2689-2694. https://doi.org/10.1128/JCM.01025-16, https://doi.org/10.1128/JCM.01025-16

Co-infection and emergence of rifamycin resistance during a recurrent *Clostridium difficile* infection

[1,2]Emma C. Stevenson, [3]Giles A. Major, [3]Robin C. Spiller, [1,2]Sarah A. Kuehne, and [1,2]Nigel P. Minton[#].

[1] Nottingham BBSRC/EPSRC Synthetic Biology Research Centre, Clostridia Research Group, School of Life Sciences, the University of Nottingham, Nottingham, UK

[2] NIHR Biomedical Research Unit in Gastrointestinal and Liver Diseases at Nottingham University Hospitals NHS Trust and The University of Nottingham

[3] QMC West Block E Floor, Queen's Medical Centre, Nottingham, NG7 2UH, UK

Corresponding author: Minton, N.P (nigel.minton@nottingham.ac.uk), Nottingham BBSRC/EPSRC Synthetic Biology Research Centre, Clostridia Research Group, School of Life Sciences, the University of Nottingham, Nottingham, UK. Tel. +44 (0)115 84 67458

Keywords: *Clostridium difficile*, relapse, rifaximin, rifampicin

Running Title: Recurrent *C. difficile* infection

Emma Stevenson: Biosciences, College of Life and Environmental Sciences, 4th floor, Geoffrey Pope. University of Exeter, Stocker Road, Exeter, EX4 4QD, UK

Abstract

Clostridium difficile (*Peptoclostridium difficile*) is a common health care associated infection with a disproportionately high incidence in elderly patients. Disease symptoms range from mild diarrhoea through to life threatening pseudomembranous colitis. Around 20% of patients may suffer recurrent disease which often requires re-hospitalisation of patients.

C. difficile was isolated from stool samples from a patient with two recurrent *C. difficile* infections. PCR-ribotyping, whole genome sequencing and phenotypic assays were used to characterise these isolates.

Genotypic and phenotypic screening of *C. difficile* isolates revealed multiple PCR-ribotypes present, and the emergence of rifamycin resistance during the infection cycle.

Understanding both the clinical and bacterial factors that contribute to the course of recurrent infection could inform strategies to reduce recurrence.

Journal of Clinical Microbiology

JCM

Journal of Clinical Microbiology

Introduction

Clostridium difficile (*Peptoclostridium difficile*) is a common health care associated infection with a disproportionately high incidence in elderly patients. Recurrent *Clostridium difficile* infection (CDI) is known to occur in approximately 20% of patients following withdrawal of treatment antibiotics (1), however, this may rise to 65% if a patient has a prior history of CDI (2). Recurring CDI not only causes distress to patients, but is also a substantial burden on the healthcare system due to the increased cost (3) associated with possible prolonged stay or re-admission of a patient to hospital and the re-administration of diagnostic tests and antibiotics (4). Collaborations between clinicians and researchers are contributing to the knowledge of how both the host and bacterium are affecting recurrent disease, in order to reduce recurrence rates, through personalised patient care regimens (5).

In this study multiple stool samples from a patient enrolled in a clinical trial (see case information) were collected and *C. difficile* was isolated. The strains isolated from these samples were then phenotypically and genotypically characterised to deduce if changes in the C. *difficile* strain genotype and phenotype could have contributed to recurrent infection.

Case

A male participant (01008) aged 85 years who had a history of chronic kidney disease and was on multiple long term medication including, bumetanide, candesartan, digoxin, simvastatin, doxazosin, ferrous fumarate and prednisolone. Three months prior to trial enrolment the participant received cefuroxime and gentamicin for a urinary tract infection. One month prior to trial enrolment he was admitted to the hospital, where he received clindamycin (both orally and intravenously) for an infected leg ulcer.

In June 2013, after being re-admitted to hospital, he was diagnosed with CDI (Table 1; sample A), was treated with metronidazole (MET) and discharged. He was diagnosed again with CDI in July (Table 1; sample B) and was again treated with MET.

In July 2013, within 5 days of cessation of successful treatment of CDI with MET (defined as cessation of diarrhoea for 2 or more days with no loose stools, assessed), he was enrolled on a randomised double blind placebo controlled clinical research trial, aimed at using Rifaximin for reducing relapse of *Clostridium* associated diarrhoea (RAPID- NCT01670149). A simplified study time line of stool sample collection is outlined in Figure 1.

The participant successfully provided one trial stool sample (Table 1; sample C). However within 10 days, suffered another diarrhoeal episode (Table 1; sample D), which was initially toxin negative as confirmed using the C. DIFF QUIK CHEK complete kit (Alere) and PCR using the BD MAX™ Cdiff kit (BD Molecular Diagnostics). However, 6 days after sample D he provided another sample (Table 1; sample E) which was toxin positive and therefore defined as relapse according to the RAPID trial protocol (onset of >3 loose bowel movements per day for at least 2 consecutive days combined with a positive toxin assay (with or without a positive *C. difficile* culture)). He was treated with MET and over 8-10 weeks provided two more trial samples (Table 1; samples F & G). Participant notes revealed that during the period when he provided more RAPID samples, he was again diagnosed with CDI (September) and treated with Vancomycin (VAN).

The participant was again admitted to hospital and treated with tazocin and subsequently suffered another CDI (Table 1; sample H), around his time of death, in January 2014 due to multiple organ failure. As this was a randomised double blind placebo controlled clinical research trial, it was not known if participant 01008 was on placebo or rifaximin therapy, at the point at which this research was undertaken.

Journal of Clinical Microbiology

Methods

A total of eight stool samples were collected from the participant (Table 1). These included diagnostic specimens from the Queens Medical Centre (QMC) microbiology department, Nottingham, that were proven *C. difficile* toxin positive by using the C. diff Quik Chek complete kit (Alere) and PCR using the BD MAX™ Cdiff kit (BD Molecular Diagnostics). *C. difficile* was cultured from ~ 300mg of stool using a previously published protocol (6). 1-20 *C. difficile* colonies were isolated per sample (Table 2).

C. difficile typing

After 48 hours (hrs) of growth on Cefoxitin Cycloserine Egg Yolk (CCEY) (6) agar, up to 20 individual *C. difficile* colonies from each stool specimen were inoculated into a single well of a 96-well plate containing 200 µL anaerobic Brain Heart Infusion (Oxoid) plus 0.1% L-cysteine (Sigma) (BHIS) broth, leaving one well blank as a control. The plate was sealed with a breathable sterile film and incubated for 24 hrs in an anaerobic workstation (Don Whitley) (CO_2:H_2:N_2 (80:10:10 vol:vol:vol,). After 24 hrs the wells were checked for turbidity. Overnight cultures were then diluted 10-fold with sterile PCR grade water into a fresh 96-well plate, sealed with film and stored at -20°C until required. One drop of 100% glycerol was added to the BHIS cultures and the plate was stored at -80°C until required. Every colony that was isolated from each stool sample was subjected to in-house ribotyping with the diluted cultures as mentioned above. PCR amplification of the 16s rRNA intergenic spacer region was carried out according to a modified protocol obtained from the former *C. difficile* ribotyping laboratory in Cardiff (Supplementary data). PCR-ribotype profiles were analysed with a QIAxcel capillary Electrophoresis machine (Qiagen) using the OL400 programme with the QX 15bp-1Kb alignment marker and the QX Size Marker 50 bp –800 bp. Individual profiles were assessed, and then one isolate from each distinct typing profile

that had been obtained from each stool sample, was re-cultured onto BHIS-CC agar and stored as a glycerol stock.

These stock strains were sent for official ribotyping via the *C. difficile* ribotyping network (CDRN) service in Leeds and used for downstream characterisation.

Phenotypic Characterisation

Growth, sporulation and toxin A and B ELISAs were performed on all isolates. (Supplementary data)

Antibiotic Susceptibility

Isolated strains were tested for antibiotic resistance to metronidazole (MET) and vancomycin (VAN), using the E-test method (Oxoid) and Rifampicin (RIF) and Rifaximin (RFX) resistance by broth dilution (see supplementary data) using a 2-fold antibiotic dilution range from 512 - 0.5 µg/ml. *C. difficile* strains 630Δ*erm*, 630 wild type (WT) and R20291 were used as controls. Guideline MIC breakpoints for RIF and RFX were taken from (7), where isolates with MICs ≥32 µg/ml were considered resistant. Intermediate resistance was defined as an MIC of 0.003–32 µg/mL (8).

Genotypic Characterisation

DNA was extracted from strains using a Phenol:Chloroform:Isoamyl alcohol (25:24:1) saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma) extraction method adapted from (9)(See supplementary data).

Whole Genome comparison

Genomic DNA was sent for Illumina sequencing using MisSeq 250-PE technology, (DeepSeq, University of Nottingham). DNA from one of the isolates from sample A (earliest RT002 isolate), and sample E (earliest RT014 identified) designated E2 were also sent for

Pacific Bioscience (PacBio) Sequencing (McGill University and Genome Québec Innovation Centre). Paired-end reads from the MiSeq runs were mapped to PacBio contigs using CLC Genomics Workbench Version 8.0.2 (Qiagen).

Concordance of PacBio and Illumina sequencing

To demonstrate the concordance of two different sequencing methods (especially over homopolymer regions), Illumina paired-end sequencing reads from isolate A and E2 were mapped back to the Pacbio reference contigs. Basic variant detection (CLC Genomics Workbench Version 8.0.2 [Qiagen]) was used to call single nucleotide variations (SNVs), insertions and deletions. All default parameters were kept the same apart from the minimum frequency setting in the coverage and count filters process. The minimum frequency setting was changed to 50% to try and capture as many high quality changes as possible.

Results

Ribotype of *Clostridium difficile* Isolates obtained from stool

During the course of infection two distinct *C. difficile* PCR-ribotypes were isolated (Table 2). The predominant PCR-ribotype occurring during the infection was RT002. Co-infection of the participant with a second PCR-ribotype (RT014), was detected in stool sample E. This sample was a diagnostic sample obtained after the participant had suffered the first relapse (Table 2). However, it cannot be deduced whether this PCR-ribotype was present in earlier samples (i.e. C or D) due to the low number of colonies obtained from the stools of these samples. The RT014 isolate, may therefore, have been present at low frequency in these samples.

Isolate Growth, Sporulation and toxin quantification

All isolates showed similar growth profiles in both BHIS and TY (data not shown) except isolate G. This isolate had a shorter stationary phase when grown in BHIS and reduced growth in TY broth. However, this did not affect sporulation and toxin titre as there were no significant differences in these phenotypes, between any of the isolates (data not shown).

Pacific Bioscience and Illumina MiSeq Sequencing of RT002 and RT014 isolates

The PacBio sequencing was able to assemble the genome of isolate A into four contigs (Table 3) and isolate E2 into one contig of 4,330,205bp. Contigs were identified by means of Blast searches using the dc-megablast option against the nt database. BLAST analysis of the one contig from isolate E2 suggests that it shares sequence similarity with the *Peptoclostridium difficile* genome assembly CD630DERM, chromosome : 1 (LN614756.1).

The individual contigs were annotated using RAST (10) and used as reference strains for Illumina read mapping of all isolates.

Over 97% of reads from seven RT002 isolates (A, B, C, D, E1, F & G) and >97% of reads from two RT014 isolates (E2 and H) mapped to the reference PacBio contigs for the corresponding PCR-ribotype.

Concordance of PacBio and Illumina sequencing

Nine SNVs were identified in all the RT002 genomes and twelve SNVs were detected when Illumina MiSeq reads were mapped back to their respective PacBio contigs. All SNVs of the RT002 isolates occurred in the first contig, which represented the main *C. difficile* chromosome. All the SNVs from both RT002 and RT014 isolates occurred in homopolymer regions of ≥4 nucleotides in length. PCR amplification and Sanger sequencing of all the regions in isolate A and E2 that contained the SNVs confirmed that they were true SNVs.

Accordingly, the reference sequences A and E2 were corrected and the sequence reads from the subsequent isolates (B-G) were remapped.

SNV detection in RT002 isolates

Basic variant detection was performed on the reads to assess whether the sequential isolates contained additional changes from the original (A) isolate (Table 4). Isolate B did not contain any additional SNVs. Isolate C contained one additional SNV. The A>G nucleotide change did not result in an amino acid change. Isolate D and E1 both contained the same four additional SNVs compared to isolate A, two of which were non-synonymous and found in *oppF* and *rpoB* (Table 4). The former encodes an oligotransport-ATP binding domain, while the latter encodes a DNA-directed RNA polymerase beta subunit. These two isolates did not contain the SNV found in isolate C. Isolate F contained five SNVs compared to A, however, these were not the same as any SNVs in previous isolates. One of the SNVs in isolate F was found in the same gene (*rpoB*), but not at the same position as in isolates D and E1. Variant detection in isolate G, produced 70 SNVs. Closer inspection of these SNVs revealed that 64 were detected at low frequency in poorly mapped regions and were probably not real. Thus, this isolate had six SNV differences compared to isolate A and B (Table 4). Five SNVs were in the same regions as isolate F with two of the SNVs, in a hypothetical protein and *rpoB*, being in the exact same location as in isolate F. (Table 4)

SNV detection in RT014 isolates

Six additional SNVs were found in isolate H compared to isolate E2. Five of these SNVs were in a gene annotated as *fliK*, but which is not actually part of the flagellar operon. Only two of these five SNVs were non-synonymous and occurred at a frequency <52% and with an average quality of <22. Closer inspection of this region revealed that the sequence quality was poor most likely due to it being repetitive, suggesting that these SNVs were not likely to

be real. Thus, only one additional T >C SNV was identified. The SNV was in an intergenic region of the genome of isolate H, at position 2562170 bp, upstream of of a gene encoding a small hypothetical protein that shows similarity to a putative membrane protein.

Isolate Antibiotic resistance

No isolate showed resistance to MET (breakpoint considered as resistant ≥2 µg/ml) or VAN (breakpoint considered as resistant ≥4 µg/ml) (data not shown). Early RT002 isolates A, B and C, showed complete susceptibility to rifampicin (RIF) and rifaximin (RFX) (Figs. S1a, S1b, S2a & S2b). However, RT002 isolates D (from the relapse sample) and E1 (post relapse sample) showed high resistance (≥ 256 µg/ml) to RIF and RFX (≥128 µg/ml), whilst RT002 isolates F and G showed intermediate resistance to both (RIF;≥4 µg/ml, RFX;≥16 µg/ml). The RT014 isolates (E2 and H) and control strains were fully susceptible to RIF and RFX (>0.5 µg/ml). (Figs. S1a, S1b, S2a & S2b).

Frequency of *rpoB* SNVs in all cultured A to G RT002 isolate samples

To try and discern whether there were two distinct populations of RIF and RFX resistant RT002 isolates, the region in *rpoB* that contained the SNVs identified in Table 4, was amplified from every isolate with a PCR-ribotype banding pattern confirmed with the QIAxcel (Table 1). The PCR amplified DNA fragment was sent for Sanger sequencing and the sequences were checked for the above SNVs. The frequency of these SNVs in each isolate is detailed in Table 5.

Discussion

The recurrent infection suffered by participant 01008 is one which is endured by up to 20% of patients suffering from CDI (1). Over a 180 day period this particular individual relapsed twice with CDI and presented with co-infection of two different PCR-ribotypes. At one

Journal of Clinical Microbiology

226 particular point during the infection theparticipant also, either acquired a RT002 isolate
227 which was resistant to rifamycins (re-infection), or the original isolate developed rifamycin
228 resistance, due to microevolution (relaspe). The combination of these factors could have
229 contributed to the persistence of this participant's infection.

230 The nature of the RAPID trial is to recruit participants at the end of their standard CDI
231 therapy, when the patient has been assessed as being resolved of CDI. The participant is then
232 started on a regimen of either RFX or placebo for four weeks during which stool samples are
233 collected. The participant is then followed up for another eight weeks during which more
234 stool is collected. As this was a randomised double blind placebo controlled clinical research
235 trial, at the point at which this research was undertaken, it was not known if participant 01008
236 was on placebo or rifaximin therapy.

237 What is clear from this data, is that even though this participant was deemed to have resolved
238 symptoms of CDI prior to trial enrolment, there was still a low level of *C. difficile* in the stool
239 (as indicated by the isolation of *C. difficile* from sample C) and in this participant it may have
240 contributed to the recurrence of disease. There is further genetic evidence to suggest that the
241 RT002 isolate found in the pre-enrolment diagnostic specimens (A and B) had persisted, at
242 least until the next sample specimen (C). According to others (11-13) genetically identical
243 strains differ by ≤2 SNVs and there was only one SNV difference between isolate A/ B and
244 C. At some point during the time between sample C and sample D, participant 01008 either
245 acquired a genetically distinct RT002 isolate (re-infection) with resistance to RIF and RFX,
246 or the original RT002 isolate evolved to become RIF and RFX resistant (≥256 µg/ml and
247 (≥128 µg/ml respectively) relapse. Prior to sample D all isolates from samples A, B and C did
248 not contain SNVs in the *rpoB* gene (Table 5), supporting the notion that participant 01008
249 was probably colonised with one population of non-resistant RT002 isolates at that time of
250 the infection. In other studies (11, 12) genetically distinct isolates are differentiated by >10

SNVs. Here isolate D differed by only four SNVs compared to isolate A,B and C and within the population of isolates from sample D (Table 5) one isolate did not contain the SNV in the *rpoB* gene. Thus, it is not possible to deduce whether this was a newly acquired isolate or whether the initial strain had mutated. It is possible that the RT002 isolate in this infection was under high antibiotic selection pressure and thus may have mutated more rapidly than others have calculated for strains not under intense selection pressure(14).

Whole genome sequencing of isolates A-H revealed that the probable cause of RIF and RFX resistance in isolate D, was a C>A SNV at position 1465bp in the *rpoB* gene, encoding a DNA-directed RNA polymerase beta subunit (Table 4). Mutations in *rpoB* have been identified in *C. difficile* (7, 8) and also occur in multi-drug resistant strains of *Mycobacterium tuberculosis* (MTB) (15) within a small 23 amino acid region from position 511-533 (16). This suggests a common mechanism by which resistance to this antibiotic occurs in multiple bacterial species.

The SNV in isolate D resulted in a Glutamine > Lysine amino acid change at position 489 of the peptide. It is known that the Glutamine residue at the corresponding position of RpoB in *Thermus aquaticus* directly binds to rifampicin (7, 17). Thus, it is likely that this SNV is directly responsible for resistance to RIF and RFX in this isolate and this may have played a role in the first relapse suffered by this participant. A subsequent stool specimen from participant 01008 revealed the presence of a genetically identical RT002 isolate (E1) to isolate D, which also shared the same SNV change in *rpoB* and was also RIF and RFX resistant (≥256 μg/ml and ≥128 μg/ml respectively). The sample also contained another ribotype (RT014 - E2). Isolate E2 was fully sensitive to RIF and RFX. If participant 01008 was on RFX therapy then it is unlikely that isolate E2 contributed to the relapse, as clearly the vegetative form of the isolate is susceptible to the rifamycins and would have been killed.

However, whether or not the isolate was present just prior to relapse, is not discernible, as it was only possible to isolate one colony from the pre-relapse sample C. The RT014 isolate (E2) may have been present in the host at low levels in the spore form, evading the effects of possible antibiotic therapy, and persisting until a time when it could germinate and grow. This hypothesis is supported by the emergence of the RT014 (H) isolate in the final stool sample which was genetically indistinct from isolate E2.

Isolates D and E1 also shared a SNV in the oligotransport-ATP binding domain of *oppF* (Table 4), which belongs to an operon of oligopeptide permease (*opp*) genes that are involved in regulating sporulation (amongst other processes) in some species of *Bacillus* and *Clostridium* (18, 19). *In vitro* sporulation studies on all isolates revealed no significant difference (data not shown) in the rate of sporulation between isolate D, E1 and all other isolates. However, this data may not be representative of *in vivo* sporulation characteristics; therefore it cannot be conclusively proven that this SNV had no effect. The *opp* operon is involved in other processes in other organisms, for example competence in *Bacillus* and *Streptococcus* species, plasmid transfer in *Enterococcus faecalis*, and the expression of virulence factors in *Bacillus thuringiensis* (18, 20). SNVs in this region may, therefore, have a yet undiscovered role in *C. difficile* virulence and could present a further avenue of research.

The next specimen provided by the patient revealed the presence of an RT002 isolate (F) with five additional SNVs compared to isolate A, B and C. Four of the SNVs were completely different to the previous isolate (E1) and isolate D. However, one SNV was again located in *rpoB*, at a different position (1475bp), resulting in an A>T change that caused an amino acid change from aspartic acid > valine at position 492 of the peptide. The substitution of this aspartic acid residue to other amino acids, including valine, has been shown to result in RIF resistance in *Staphylococcus aureus* (7, 21). Unlike the RpoB amino acid changes in isolates

300 D and E1, the RpoB amino acid change in isolate F, apparently conferred only intermediate
301 resistance to RIF(≥ 4 µg/ml) and RFX (≥µg/ml). The SNV at this position was also shared
302 by the final RT002 isolate found in sample G, which showed the same level of resistance to
303 RIF and RFX. Whether or not this level of resistance would be sufficient to contribute to the
304 persistence of the organism in the colon is unclear, but there is clearly selective pressure for
305 this mutation occurring either in the host or within the *C. difficile* population found at the
306 QMC in Nottingham.

307 The presence of a SNV in the same gene (*rpoB*), but at different positions within the RT002
308 isolates, supports the notion that two distinct subpopulations of RIF and RFX resistant RT002
309 isolates could have co-existed in this participant. Further sequencing of this region in sample
310 E RT002 isolates, revealed that they all shared the same C>A SNV. However, one of the
311 fifteen sample F isolates cultured (Table 5) contained the C>A SNV found in sample E
312 isolates and not the A>T SNV found in the remaining F isolates, indicating that two sub-
313 populations of RT002 isolates could have potentially co-existed at this point. Amongst the
314 sample G isolates, there were equal population of isolates with and without any SNVs in
315 *rpoB*. This either indicates the persistence of the initial susceptible isolate in the gut, or that at
316 the time sample G was taken the population was in a transient state of mutation.

317 It is poignant to note that other studies have documented cases of *C. difficile* rifamycin
318 resistance after chaser therapies using rifaximin (8, 22, 23). One of these studies has linked
319 this resistance to mutations in RpoB (8) that have been identified by others (7). The study by
320 Curry *et al*. (8) indicates that in their study population, more than one-third of isolates were
321 resistant to rifaximin and this is something which could be looked at in a wider population of
322 participants on the RAPID trial, as it may prompt clinicians to alter dosing regimen if the
323 therapy was approved for use in patients suffering recurrence.

Conclusion

This case study has presented insight in to the course of recurrent infection caused by *C. difficile*. In this case it was difficult to ascertain whether, in this particular individual, the *C. difficile* strain was evolving. However, it did reveal the possible presence of multiple isolates with SNVs causing distinct fitness advantages. The fact that this participant was enrolled in a trial to investigate the use of RFX to prevent recurrence suggests that we should be monitoring mutations in the *rpoB* gene more closely in the isolates from trial patients, as a side effect of this therapy could be the increased selection for RFX resistant *C. difficile* strains. This is of importance to clinicians as it may directly impact the antibiotic regimen they use to treat their patient.

The advent of high throughput technologies will allow for more in depth screening of samples to elucidate the true genetic fingerprint of the isolates found during infection. When coupled with in-depth microbiome analysis of the host, this may allow researchers to more fully comprehend the overall picture of recurrent infection and in turn this translate this information to clinicians, in order to manage 'at risk' patients more effectively and reduce the morbidity and economic burden of *C. difficile* within the healthcare system.

Funding

The RAPID trial is facilitated by the National Institute for Health Research (NIHR) Nottingham Digestive Diseases Biomedical Research Unit at Nottingham University Hospitals (Trial number NCT01670149) under the grant number: PB-PG-0110-21041 and also grant 20022.

Conflict of Interest

The authors declare that there is no conflict of interest

Acknowledgments

The authors would like to thank the Dr Mathew Diggle and members of the Microbiology department at the QMC for their help with sample acquisition. We would also like to thank the staff of the CDRN in Leeds for PCR-ribotyping sample isolates. The authors would also like to acknowledge the University of Nottingham's sequencing facility (DeepSeq) and the Génome Québec/Genome Canada-funded Innovation Centre for sequencing of *C. difficile* strains for this study. This is a summary of independent research supported by the National Institute for Health Research (NIHR)'s Nottingham Digestive Diseases Biomedical Research Unit. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

References

1. **Johnson S.** 2009. Recurrent *Clostridium difficile* infection: A review of risk factors, treatments, and outcomes. Journal of Infection **58:**403-410.
2. **Hu MY, Katchar K, Kyne L, Maroo S, Tummala S, Dreisbach V, Xu H, Leffler DA, Kelly CP.** 2009. Prospective Derivation and Validation of a Clinical Prediction Rule for Recurrent *Clostridium difficile* Infection. Gastroenterology **136:**1206-1214.
3. **Ghantoji SS, Sail K, Lairson DR, DuPont HL, Garey KW.** 2010. Economic healthcare costs of *Clostridium difficile* infection: a systematic review. Journal of Hospital Infection **74:**309-318.
4. **Dubberke E, Wertheimer A.** 2009. Review of current literature on the economic burden of *Clostridium difficile* infection. Infect Control Hosp Epidemiol **30:**57 - 66.
5. **Barbut F, Rupnik M.** 2012. Editorial Commentary: 027, 078, and Others: Going Beyond the Numbers (and Away From the Hypervirulence). Clinical Infectious Diseases **55:**1669-1672.

6. **Lister M, Stevenson E, Heeg D, Minton NP, Kuehne SA.** 2014. Comparison of culture based methods for the isolation of *Clostridium difficile* from stool samples in a research setting. Anaerobe **28:**226-229.

7. **O'Connor JR, Galang MA, Sambol SP, Hecht DW, Vedantam G, Gerding DN, Johnson S.** 2008. Rifampin and Rifaximin Resistance in Clinical Isolates of *Clostridium difficile*. Antimicrobial Agents and Chemotherapy **52:**2813-2817.

8. **Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, William PA, Harrison LH.** 2009. High Frequency of Rifampin Resistance Identified in an Epidemic *Clostridium difficile* Clone from a Large Teaching Hospital. Clinical Infectious Diseases **48:**425-429.

9. **Sambrook J, Russell D, W.** 2001. Molecular Cloning: A labratory Manual, Third Edition ed, vol 1. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

10. **Aziz R, Bartels D, Best A, DeJongh M, Disz T, Edwards R, Formsma K, Gerdes S, Glass E, Kubal M, Meyer F, Olsen G, Olson R, Osterman A, Overbeek R, McNeil L, Paarmann D, Paczian T, Parrello B, Pusch G, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics **9:**75.

11. **Eyre DW, Cule ML, Wilson DJ, Griffiths D, Vaughan A, O'Connor L, Ip CLC, Golubchik T, Batty EM, Finney JM, Wyllie DH, Didelot X, Piazza P, Bowden R, Dingle KE, Harding RM, Crook DW, Wilcox MH, Peto TEA, Walker AS.** 2013. Diverse Sources of *C. difficile* Infection Identified on Whole-Genome Sequencing. New England Journal of Medicine **369:**1195-1205.

12. **Eyre DW, Babakhani F, Griffiths D, Seddon J, Del Ojo Elias C, Gorbach SL, Peto TEA, Crook DW, Walker AS.** 2014. Whole-Genome Sequencing Demonstrates That Fidaxomicin Is Superior to Vancomycin for Preventing Reinfection and Relapse of Infection With *Clostridium difficile*. Journal of Infectious Diseases **209:**1446-1451.

13. **Mac Aogáin M, Moloney G, Kilkenny S, Kelleher M, Kelleghan M, Boyle B, Rogers TR.** 2015. Whole-genome sequencing improves discrimination of relapse from reinfection and

identifies transmission events among patients with recurrent *Clostridium difficile* infections. Journal of Hospital Infection **90:**108-116.

14. **Didelot X, Walker AS, Peto TE, Crook DW, Wilson DJ.** 2016. Within-host evolution of bacterial pathogens. Nat Rev Micro **14:**150-162.

15. **Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S.** 2012. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nat Genet **44:**106-110.

16. **Telenti A, Imboden P, Marchesi F, Matter L, Schopfer K, Bodmer T, Lowrie D, Colston MJ, Cole S.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. The Lancet **341:**647-651.

17. **Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA.** 2001. Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase. Cell **104:**901-912.

18. **Edwards AN, Nawrocki KL, McBride SM.** 2014. Conserved Oligopeptide Permeases Modulate Sporulation Initiation in *Clostridium difficile*. Infection and Immunity **82:**4276-4291.

19. **Nawrocki KL, Edwards AN, Daou N, Bouillaut L, McBride SM.** 2016. CodY-dependent Regulation of Sporulation in *Clostridium difficile*. Journal of Bacteriology doi:10.1128/jb.00220-16.

20. **Moraes PMRO, Seyffert N, Silva WM, Castro TLP, Silva RF, Lima DD, Hirata R, Silva A, Miyoshi A, Azevedo V.** 2014. Characterization of the Opp Peptide Transporter of *Corynebacterium pseudotuberculosis* and Its Role in Virulence and Pathogenicity. BioMed Research International **2014:**7.

21. **Murphy CK, Mullin S, Osburne MS, van Duzer J, Siedlecki J, Yu X, Kerstein K, Cynamon M, Rothstein DM.** 2006. In Vitro Activity of Novel Rifamycins against Rifamycin-Resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy **50:**827-834.

22. **Johnson S, Schriever C, Galang M, Kelly CP, Gerding DN.** 2007. Interruption of Recurrent *Clostridium difficile*-Associated Diarrhea Episodes by Serial Therapy with Vancomycin and Rifaximin. Clinical Infectious Diseases **44:**846-848.

23. **Johnson S, Schriever C, Patel U, Patel T, Hecht DW, Gerding DN.** 2009. Rifaximin Redux: Treatment of recurrent *Clostridium difficile* infections with Rifaximin immediately post-vancomycin treatment. Anaerobe **15:**290-291.

Table 1- Description and date of sample collection for participant 01008 on the RAPID trial

ID	Specimen	Sample collection date
A	**1st diagnostic sample***	21/6/13
B	2nd diagnostic sample*	1/7/13
C	**Week 0 (visit 1) RAPID sample**	9/7/13
D	Relapse sample 1	19/7/13
E	Diagnostic sample post relapse sample 1*	27/7/13

F	**Week 4 (visit 3) RAPID sample**	7/8/13
G	**Week 12 (Visit 5) RAPID sample**	2/10/13
H	Relapse sample after week 12 (visit 5) RAPID sample	4/1/14

*Samples obtained from Queens Medical Centre microbiology department

Table 2- PCR-ribotype of isolates obtained from participant 01008 stools samples

ID	Specimen	Sample collection date	Number of Colonies obtained/typed*	PCR-ribotype of isolate
A	**1st diagnostic sample**	21/6/13	20/8	002
B	2nd diagnostic sample	1/7/13	20/20	002
C	**Week 0 (visit 1) RAPID sample**	9/7/13	1/1	002
D	Relapse sample 1	19/7/13	8/5	002
E	Diagnostic sample post relapse sample 1	27/7/13	20/20	E1-002 (7) E2- 014 (13)
F	**Week 4 (visit 3) RAPID sample**	7/8/13	15/14	002

G	**Week 12 (Visit 5) RAPID sample**	2/10/13	20/20	002
H	Relapse sample after week 12 (visit 5) RAPID sample	4/1/14	20/20	014

*In house capillary electrophoresis typing using a QIAxcel. Numbers in brackets of column five represent the frequency of that PCR-ribotype pattern

Table 3: PacBio Contig Assembly information from RT002 isolate A.

Contig	PacBio BLAST identification	Contig Size
1	FN668944.1, *Clostridium difficile* BI9 chromosome	4,207,942bp
2	LN681537.1, *Clostridium* phage phiCD211, complete genome	140,450bp
3	FN668942.1, *Clostridium difficile* BI1 plasmid pCDBI1, complete sequence	65,380bp
4	GU949551.1, *Clostridium* phage phiCD6356, complete genome	52,160bp

483

484

485

Strain	Reference Position	Type	Reference	Allele	Count	Coverage	Frequency	Average quality	Coding region change	Amino acid change
C	319864	SNV	A	G	194	201	96.52	33.44	Choline binding protein A:c.6498A>G	
D	147783	SNV	A	T	189	192	98.44	36.37	Aminobenzoyl-glutamate transport protein:c.210T>A	
D	556338	Deletion	A	-	177	177	100.00	31.07	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA	p.Gln160fs
D	2974068	SNV	G	A	210	233	90.13	36.21		
D	3762293	SNV	C	A	189	191	98.95	36.61	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C>A	p.Gln489Lys
E1	147783	SNV	A	T	195	198	98.48	34.89	Aminobenzoyl-glutamate transport protein:c.210T>A	
E1	556338	Deletion	A	-	146	149	97.99	32.74	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA	p.Gln160fs
E1	2974068	SNV	G	A	151	211	71.56	36.64		
E1	3762293	SNV	C	A	161	161	100.00	36.15	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C>A	p.Gln489Lys
F	1181867	SNV	G	T	172	174	98.85	33.01	FIG00512976: hypothetical protein:c.722C>A	p.Thr241Asn
F	1861424	SNV	A	G	158	168	94.05	31.75		
F	1861431	SNV	G	A	171	172	99.42	35.88		
F	2676955	SNV	A	C	231	233	99.14	35.90		
F	3762308	SNV	A	T	204	207	98.55	34.08	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A>T	p.Asp492Val

Journal of Clinical Microbiology

G	1181870	SNV	G	T	33	33	100	37.12	FIG00512976: hypothetical protein:c.722C>A	p.Thr241Asn
G	1861429	SNV	A	G	21	21	100	32.95		
G	1861436	SNV	G	A	22	22	100	37.64		
G	2676961	SNV	A	C	12	12	100	38.67		
G	3762308	SNV	A	T	143	174	82.18	36.96	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A>T	p.Asp492Val
G	3040149	SNV	C	A	21	33	63.64	38.24	FIG00534171: hypothetical protein:c.13G>T	p.Glu5*

Table 4- Additional SNVs in genome of sequential RT002 isolates

Table 5. Frequency of all cultured RT002 isolates with and without *rpoB* SNVs

ID	Specimen	Number of Colonies typed	SNV present*
A	**1st diagnostic sample**	8	-
B	2nd diagnostic sample	20	-
C	**Week 0 (visit 1) RAPID sample**	1	-
D	Relapse sample 1	5	+ (4) [C>A] - (1)
E	Diagnostic sample post relapse sample 1	7	+
F	**Week 4 (visit 3) RAPID sample**	14	+ [A>T (13)] + [C>A (1)]
G	**Week 12 (Visit 5) RAPID sample**	20	+ (10) [A>T] - (10)

* (-): absence of SNV, (+): presence of SNV. Numbers in brackets are the frequency of the isolates with or with the SNV. Square brackets identify which SNV was present.

498

499

500

501

502

503 **Figure 1-** A simplified study timeline indicating the collection of stool samples for the participants involved in

504 the RAPID trial.

505

Journal of Clinical Microbiology

JCM

Journal of Clinical Microbiology